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ras Activation in Human Tumors and in Animal Model Systems

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Environmental agents such as radiation and chemicals are known to cause genetic damage. Alterations in a limited set of cellular genes called proto-oncogenes lead to unregulated proliferation and differentiation. We have studied the role of the *ras* gene family in carcinogenesis using two different animal models. In one case, thymic lymphomas were induced in mice by either gamma or neutron radiation, and in the other, keratoacanthomas were induced in rabbit skin with dimethylbezanthracene. Human keratoacanthomas similar to the ones induced in rabbits were also analyzed. We found that different types of radiation such as gamma rays and neutrons, induced different point mutations in *ras* genes. A novel K-*ras* mutation in codon 146 has been found in thymic lymphomas induced by neutrons. Keratoacanthomas induced in rabbit skin by dimethylbenzanthracene show a high frequency of H-*ras*-activated genes carrying a mutation in codon 61. The same is observed in human keratoacanthomas, although mutations are in both the 12th and the 61st codons of the H-*ras* gene. H-*ras* activation is less frequent in human squamous cell carcinomas than in keratoacanthomas, suggesting that *ras* genes could play a role *in vivo* in differentiation as well as in proliferation.

Introduction

Cell growth is a carefully regulated process that responds to specific needs of organismic development. However, occasionally, the sophisticated mechanisms of control that regulate cell multiplication break down and a cell begins to proliferate, giving rise to a tumorous growth. Both benign and malignant tumors represent abnormal cell growth, although they are completely different in terms of their properties of invasiveness and spread.

Changes in the DNA of somatic cells are the genetic events that transform a normally regulated cell into one that grows without responding to controls. These changes have been recently assigned to a group of genes called proto-oncogenes. When they are activated these genes are called oncogenes, whose products cause the inappropriate cell growth. One group of such oncogenes constitutes the *ras* gene family. Members of the *ras* gene family (H-, K-, and N-*ras*) are mutated in approximately 20% of all human tumors, making them the most commonly activated oncogenes (*1,2*). *ras* Genes code for similar proteins of molecular weight 21,000 (188 amino acids). There is a striking correlation between the tissue type affected in experimental tumors and the type of *ras* gene activated. All epithelial-derived tumors (skin, breast, liver) have H-*ras* activated, while mesenchymal tumors (lymphomas, fibrosarcomas, renal mesenchymal) have either K-*ras* or N-*ras* activated (*3–5*).

Activation of *ras* oncogenes has been shown to occur via point mutations in a limited number of codons (most frequently codons 12, 13, and 61). These codons are believed to code for amino acids that are important for p21 activity. *ras* Genes activated by somatic mutations *in vivo* have been most frequently detected in codons 12 or 61 (*6*).

In this report we outline the result obtained on *ras* activation in tumors different in *a*) species (mouse, rabbit, human), *b*) type of tumor (lymphomas, keratocanthomas), *c*) inducing agent (radiations, chemicals, spontaneous), and *d*) tumor agressivity (malignant and benign). Different members of the *ras* gene family are reproducibly activated in the different tumors. Molecular analysis in these models indicates that *ras* activation interferes both with cell proliferation and differentiation, but other genetic changes are necessary for tumor development.

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Methods to Detect *ras* Mutations

We have used two assays that can demonstrate the existence of activated *ras* oncogenes in tumors and which demonstrate that transforming activity is associated with the genes. These methods, the nude mouse assay and focus-forming assay, take advantage of the fact that activated *ras* genes "transform" NIH 3T3 cells; that is, the cells lose the property of contact inhibition and acquire a rounded morphology. In these assays, tumor DNA is transfected into NIH 3T3 cells. In the focus-forming assay, the cells are cultured in 5% calf serum. Under these conditions, *ras*-transformed cells have a growth advantage over normal cells and form "foci" of phenotypically transformed cells over the monolayer of normal cells. In the nude mouse assay, tumor DNA is co-transfected with a G418-resistance gene as a dominant, selectable biochemical marker. Cells that take up the foreign DNA will survive in the presence of the antibiotic G418. After 2 weeks, cells are inoculated into nude mice. Tumor formation is associated with the presence of transforming DNA sequences in the injected cells (*7*). Molecular analysis of foci DNA and nude mouse tumor DNA is then used to determine the oncogene (*ras* or non-*ras*) that is present. The transforming sequences are detected by Southern blot analysis.

Two methods were used to identify point mutations in *ras* genes in primary or nude mouse tumors. Prior to assaying for a *ras* mutation, the exon containing the possible *ras* mutation was amplified by the polymerase chain reaction (PCR) (*8–10*). In the first assay, the amplified samples were then affixed to nylon membranes using a slot blot apparatus, and the membranes were hybridized to ^{32}P-labeled oligonucleotides specific for the relevant point mutations. The membranes were washed in conditions in which no labeled probe remained bound to slots containing PCR-amplified normal brain DNA. In the second assay, the amplified DNA fragments were sequenced using a modification of the Sanger dideoxy chain terminator method (*11,12*).

Comparison between Different Forms of Ionizing Radiation

Interaction of Ionizing Radiation with Cells and Molecules

Among the environmental agents that are potentially carcinogenic are different forms of ionizing radiation. Ionizing radiation can be classified as being particulate or electromagnetic or as being densely or sparsely ionizing. We have compared the carcinogenic effects of gamma radiation, a sparsely ionizing electromagnetic radiation, with neutrons, a densely ionizing particulate radiation. Because of the different properties of these types of radiation, they deliver energy to intracellular target molecules in different ways (*13*). Neutrons directly interact with intranuclear target molecules, while gamma rays transfer energy to orbital electrons of several intracellular molecules, mainly water, creating free radicals that chemically react with DNA.

Both neutron and gamma irradiation are potential carcinogens, as demonstrated by the fact that both can transform cells *in vitro* (*14–16*) and induce tumors, including thymic lymphomas, in animals (*17,18*). One goal of this study was to determine whether the differences in qualities of these different forms of radiation might result in tumors with a different pattern of *ras* gene mutations. To that aim, we induced tumors with neutrons and compared the results with previous data obtained from tumors induced by gamma radiation.

Induction of Mouse Thymic Lymphomas with Neutrons

An optimal dose of neutron radiation for efficiently inducing thymic lymphomas in female RF/J mice (age 4–7 weeks) was determined by testing several different doses (0.8, 1.0, 2.0, and 3.0 Gy) of 0.44 MeV neutrons administered at one time as whole body irradiation. The most effective dose was 1.0 Gy, with 71% of 34 mice that received this dose developing thymic lymphomas (mean latency of induction of 170 ± 23 days). A dose of 2.0 or 3.0 Gy of neutrons resulted in radiation sickness and early death, and only 1 of 7 (14%) of mice that received 0.8 Gy of neutrons developed a thymic lymphoma.

Activating *ras* Mutations Detected in Neutron-Induced Thymic Lymphomas

Two assays, the nude mouse assay and differential oligonucleotide hybridization to PCR-amplified DNA, were independently used to detect activated *ras* genes in the neutron-induced thymic lymphomas (*8,19,20*). Both assays were used because the nude mouse assay detects the transforming activity of a gene, while differential oligonucleotide hybridization detects specific mutations in the gene.

A total of six neutron-induced thymic lymphomas scored positive in the nude mouse assay. Southern blot analysis of nude mouse tumor DNA showed that of these six thymic lymphomas which scored positive in the nude mouse assay, three contained an activated K-*ras* gene, one contained an activated N-*ras* gene, and two did not contain an actived H-, K-, or N-*ras* gene.

The second assay, differential oligonucleotide hybridization to PCR-amplified DNA, only detects the specific mutations that the oligonucleotide probes are designed to detect. The first two exons of K- and N-*ras* were separately amplified by PCR in each of the thymic lymphomas using primers that have been described elsewhere (*10*). We decided to screen these tumors for all possible missense mutations in codons 12, 13, and 61 since these are the locations where most activating *ras* mutations and all activating *ras* mutations in murine thymic lymphomas have been detected (*6,21,22*). Of the 24 thymic lymphomas screened in this assay, 3 were

shown to contain a mutated *ras* gene. No two tumors contained the same mutation. Two of the mutations were in codon 12 of K-*ras* (GGT to TGT and GGT to GTT), and the other mutation was in codon 61 of N-*ras* (CAA to AAA) (Fig. 1). These three thymic lymphomas had scored positive in the nude mouse assay. Differential oligonucleotide hybridization to PCR-amplified DNA confirmed that each nude mouse tumor derived from a thymic lymphoma containing a *ras* mutation contained the same *ras* mutation as the thymic lymphoma from which it was derived.

One neutron-induced lymphoma and the three nude mouse tumors derived from it that contained an activated K-*ras* gene did not contain any of the mutations screened for by differential oligonucleotide hybridization. In order to determine the mutation responsible for activating this K-*ras* gene, each of the five coding exons of K-*ras* (1,2,3,4A, and 4B) from one of the nude mouse tumors was sequenced after PCR amplification. The only mutation detected, an ACA-for-GCA substitution, was located in codon 146. This mutation, which results in the substitution of the amino acid threonine for the normal alanine, has not been detected in any other system. Differential hybridization of oligonucleotides to PCR-amplified DNA confirmed that the mutation in codon 146 was present in the thymic lymphoma (Fig. 1).

Comparison between Neutron and Gamma Radiation in *ras* Mutation Induction

To determine the frequency of the codon 146 mutation in thymic lymphomas induced by gamma radiation and neutron radiation, we screened DNA samples from the 24 neutron-induced thymic lymphomas and from 25 gamma-radiation-induced thymic lymphomas for the codon 146 mutation using differential oligonucleotide hybridization. All tumors had been induced in RF/J mice and had been previously screened for mutations in codons 12, 13, and 61 of K- and N-*ras* (*21*). None of these thymic lymphomas contained a point mutuation in the first base of codon 146.

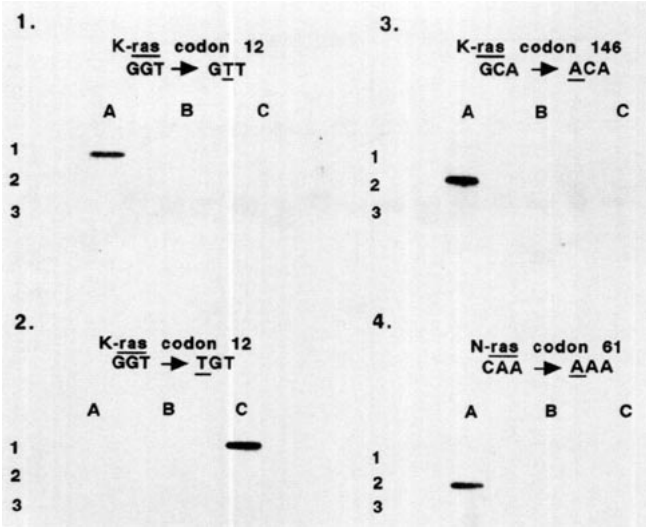

FIGURE 1. Slot blots of neutron-induced tumor DNA hybridized to oligonucleotide probes specific for single point mutations. Each panel consists of eight slots containing neutron-induced tumor DNA and one slot containing normal RF strain mouse brain DNA. In each panel, a different tumor is positive for the indicated mutation. Normal brain DNA samples are located in slots 3c of panels 1 and 4 and 3A of panels 2 and 3.

Unlike gamma radiation, which preferably induces one particular point mutation, neutron radiation induces a variety of *ras* point mutations (Table 1). In a previous report from our laboratory, 24% (9 of 37) of gamma radiation-induced thymic lymphomas contained an activated *ras* gene (*21*). Eighty-nine percent of the tumors (8 of 9) contained an activated K-*ras*, and 87.5% (7 of 8) of these had a GGT to GAT point mutation in codon 12 of K-*ras* (*21*). In comparison to gamma-ray-induced tumors, a smaller proportion of neutron-induced tumors contained an activated *ras* gene (17% versus 24%). The spectra of *ras* mutations induced by the two forms of radiation were also different. Unlike gamma radiation, neutrons have not lead to a preponderance of any one particular mutation and have not induced any tumors with a GAT for GGT substitution in K-*ras* codon 12, the most common mutation detected in gamma-ray-induced tumors. This difference in *ras* mutation spectra was found to be statistically significant.

Several hypotheses could explain the different spectra of *ras* mutations induced by different forms of radiation. Biological selection alone cannot explain the preponderance of one mutation induced by gamma radiation since many other mutations, some of which have been induced by neutrons radiation, are biologically active.

DNA conformation and DNA-protein interactions could make the second base of codon 12 susceptible to the effects of gamma radiation. Gamma radiation damages DNA indirectly through the production of free radicals (*23*), and the conformation of DNA and DNA-protein interactions could affect the damage created by free radicals (*24*).

Relative inefficiency in repairing a gamma-radiation-induced lesion at one particular base might account for the high frequency of a gamma-radiation-induced mutation at that site. While it is known that the effective-

Table 1. Comparison between activating mutations in neutron- and gamma-radiation-induced tumors.

Activating mutations	Gamma rays[a]	Neutrons
K12 GGT → GAT	7 (19%)	0
K12 GGT → TGT		1 (4%)
K12 GGT → GTT		1 (4%)
K146 GCA → ACA		1 (4%)
N61 CAA → AAA		1 (4%)
N12 GGT → GAT	1 (3%)	0
Total tumors	37	24
Total tumors with an activated *ras* gene	9 (24%)	4 (17%)

[a]The activating mutation was not determined in one tumor containing an activated K-*ras* gene. These results are summarized from Sloan et al. (*10*) and Diamond et al. (*21*).

ness of the *Uvr*ABC excision repair system of *Escherichia coli* is affected by the nucleotides near a damaged base (*25*) and that DNA damage produced by gamma radiation is often repaired in mammalian cells (*23*), it is unknown whether mammalian repair enzymes are affected by the DNA sequence near a damaged base. Neutron-induced damage may be more difficult to repair because neutrons are thought to produce dense clusters of lesions in DNA molecules (*26*).

Alternatively, neutrons may induce DNA damage that is qualitatively different from gamma-ray-induced lesions, and these neutron-induced lesions may be inefficiently repaired by cellular repair enzymes. Difficulty in repairing neutron-induced DNA lesions regardless of the location of the lesion could result in a variety of mutations in neutron-induced tumors.

Biological Activity of *ras* Mutated at Codon 146

To verify that the novel form of K-*ras* mutated in codon 146 is an active oncogene, we tested this gene in the NIH 3T3 focus-forming assay. Nude mouse tumor DNA samples were used in the focus-forming assay, and normal thymus DNA was used as the negative control. While the K-*ras* gene mutated in codon 146 was active in the focus-forming assay, it was less efficient than K-*ras* activated by a GAT for GGT substitution in codon 12 or by a GAT for GGT substitution in codon 13 (0.9 foci/μg versus 0.29 foci/μg versus 0.18 foci/μg, respectively). These differences were not due to differences in the number of copies of activated K-*ras*, since densitometric analysis of Southern blots showed that the nude mouse tumor containing the K-*ras* codon 146 mutated gene contained more copies of integrated K-*ras* than did the other tumors used in the focus-forming assay.

In comparison to the activity of other mutations in the NIH 3T3 focus-forming assay, the reduced activity of the *ras* gene mutated in codon 146 suggests that this gene has weaker transforming activity than do other activating mutations. The latencies of the nude mouse tumors induced by *ras* genes activated by different mutations also suggest that the codon 146 mutation confers less transforming activity on the *ras* gene than do other mutations. The latency of the nude mouse tumors containing the K-*ras* gene mutated at codon 146 was 21.7 ± 3.5 days; the latency of the *ras* genes mutated in codons 12 or 61 were 16.5 ± 1.6 days. This difference was statistically significant. In agreement with our findings, a recent report showed that an *in vitro* mutagenized H-*ras* gene with a mutation in codon 146 had less transforming activity than did a *ras* gene with a codon 12 mutation (*27*).

A Ser-Ala-Lys consensus sequence at codons 145 to 147 of the N-, K-, and H-*ras* genes of mammals and in the *ras* genes of several other eukaryotes (*6*) implies that this region has functional importance. X-ray analysis of crystallized p21 predicts that this region of the molecule interacts with the purine component of GTP and GDP (*28,29*). Furthermore, increased guanine nucleotide exchange rates have been measured in the protein encoded by an H-*ras* gene mutated at codon 146 by *in vitro* mutagenesis (*27*).

ras Activation in Chemically Induced Rabbit Keratoacanthomas

Skin carcinogenesis is a convenient model because tumor development can be easily monitored. We took advantage of this by inducing keratoacanthomas in rabbit skin with the cyclic hydrocarbon dimethylbenzanthracene (DMBA). Keratoacanthomas are benign tumors that can be induced by repeated applications of DMBA on the internal face of rabbit ears and spontaneously regress (*30,31*). Therefore, keratoacanthomas offer a unique model for the identification of oncogenes that are not sufficient to maintain tumor growth. DNAs from different tumors were transfected into NIH 3T3 cells and tested for the presence of transforming genes in the focus-forming assay, as described above. About 60% (10 out of 17) scored positive in this assay. Southern blot hybridizations with a rat H-*ras* probe demonstrated that all of the transformants contained extra copies of the rabbit H-*ras* gene (Fig. 2).

The transforming p21 present in keratoacanthoma transformants was detected by immunoprecipitation with the monoclonal antibody Y13-259, which detects *ras* proteins from different species (*32*). The transforming H-*ras* protein had a faster electrophoretic mobility than the normal protein (Fig. 3). This suggested that the activating mutation was in codon 61 because mutations in this codon give rise to faster forms of p21,

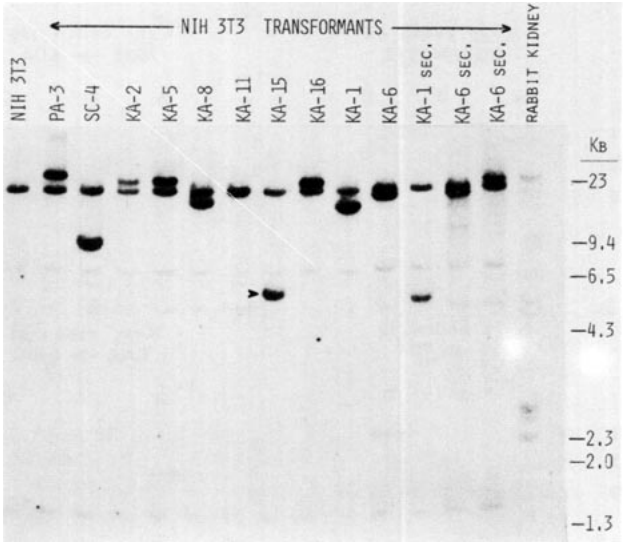

FIGURE 2. Identification of H-*ras* as the transforming oncogene of rabbit keratoacanthomas. DNAs from the corresponding NIH 3T3 transformants were digested with EcoRI and analyzed by Southern blot using the rat v-H-*ras* gene as a probe (BS9). The arrow indicates the extra band of the transformant used to clone the gene.

FIGURE 3. Immunoprecipitation of *ras* proteins from rabbit keratoacanthoma transformants. The cells were labeled with ^{35}S-Met, lysed, and immunoprecipitated with a monoclonal antibody. The samples are: two NIH 3T3 transformants from two rabbit keratoacanthomas (*A,B*), rabbit fibroblasts R9ab (*C*), NIH 3T3 (*D*), human fibroblasts (*E*), and NIH 3T3 transformed with H-*ras* mutated in codon 12 (T24 transformant) (*F*).

whereas mutations in codon 12 generate proteins with a slightly decreased mobility in polyacrilamide gels (*33*).

To identify the mutation responsible for H-*ras* activation in rabbit keratoacanthomas, we cloned the transforming gene from a transformant. The sequence of the second exon of the gene revealed a mutation in the 61st codon of the protein. This is an A:T to T:A transversion that changes the codon from CAG (Gln) to CTG (Leu). The mutations present in the other rabbit keratoacanthomas were analyzed by polymerase chain reaction amplification and oligonucleotide mismatch hybridization (*34*). This technique revealed that 90% of the keratoacanthomas contained the same mutation in codon 61 (*35*). Other types of tumor induced by DMBA contained activated *ras* genes with mutations in codon 61. This includes mouse skin carcinomas (*4*) and papillomas (*36*) and rat mammary tumors (*37*). The reason for this preferential mutation in codon 61 by DMBA might be due to carcinogen specificity. The finding of H-*ras* activation in tumors that spontaneously regress such as keratoacanthomas indicates that this oncogene is not sufficient to induce a frank malignant phenotype.

Comparison between H-*ras* Activation in Human Benign and Malignant Tumors of the Skin

The data presented above show that benign and self-regressing tumors induced in rabbits with DMBA treatment contain an activated H-*ras* oncogene. To find out if the animal model was reflecting the scenario occurring in humans, as well as to understand the specific role of *ras* activation in the early stages of tumorigenesis, we studied the frequency of H-*ras* mutations in keratoacanthomas and squamous cell carcinomas, two types of tumors with clearly different biological behaviors.

The analysis to determine the presence of activated *ras* genes in those tumors required the use of a substantial number of samples available only in the form of formalin-fixed, paraffin-embedded tissue. It has been previously shown that by PCR amplification, this material can be screened for the presence of viral sequences (*38*) or *ras* mutations (*39*). The first and second exons of the H-*ras* gene were amplified *in vitro* from 50 samples of keratoacanthomas and 50 samples of squamous cell carcinomas using primers that have been described elsewhere (*9*). One-tenth of the amplification reaction mixture from each tumor as well as normal control DNA was slot-blotted onto a nylon membrane and hybridized with a mixture of oligonucleotide probes specific for any mutation occurring in either the 12th or the 61st codon of H-*ras*. As amplification from formalin-fixed and paraffin-embedded tissues is uneven, we measured the signal produced when the slot blot is hybridized with a probe that gives equal signals with the normal and mutated H-*ras* alleles (data not shown). A second slot blot was then preparerd, correcting for differences in PCR amplificaton and hybridized with the oligonucleotide probes specific for mutations in codon 12 and 61.

The results are shown in Table 2. Four mutations in codon 12 and 10 mutations in codon 61 were identified in keratoacanthomas, representing an incidence of 30% of H-*ras* mutations among the benign tumors analyzed. Three mutations in codon 12 and 3 in codon 61 were identified in squamous cell carcinomas, representing a significantly lower incidence (13%) of H-*ras* mutations in these malignant skin tumors.

To validate further the data obtained in the PCR-oligonucleotide hybridization analysis, we sequenced some of the DNA fragments amplified from paraffin blocks, although the use of the same filter for the different probes is in itself a demonstration of the selectivity of washing conditions used. The nucleotide sequences of the region around codon 12 of DNA amplified from normal skin, from a freshly obtained keratoacanthoma, and from a paraffin-embedded tumor are shown in Figure 4.

Discussion

The experiments presented in the skin tumors are an indication of the usefulness of model systems to study human disease. It is important to note that activation of H-*ras* in this system seems to be an early event, as it can be detected in benign tumors. In this particular model system of rabbit keratoacanthomas, not only the activated oncogene is present in the benign growth, but it seems to be unable to carry the tumors forward toward malignant phenotype. This regressing behavior of the keratacanthomas seems to be independent of an inmunological reaction in both rabbits (*40*) and humans (*41*). On the contrary, the regression seems to be preceded by an intense process of differentiation that produces a marked keratinization that results in the formation of a keratin plug that eventually falls off when the regression is almost completed.

This observation, together with the results presented here in humans, where squamous cell carcinomas have a significantly lower frequency of H-*ras* activation than keratoacanthomas, is consistent with the notion that H-*ras* might be playing a role in differentiation during the regressing phase of this tumor. This would explain why H-*ras* is less frequent in squamous cell carcinomas since

Table 2. Comparison between activation in codons 12 and 61 of the H-*ras* gene from human skin tumors.

Codon	Type of mutation	Number of mutations	
		Keratoacanthoma	Squamous cell carcinoma
H-*ras* codon 12	Cys (TGC)	2	2
	Ser (AGC)	2	0
	Asp (GAC)	1	1
H-*ras* codon 61	Lys (AAG)	1	0
	Arg (CGG)	1	0
	Leu (CTG)	9	3
Frequency of H-*ras* mutations		30%	13%

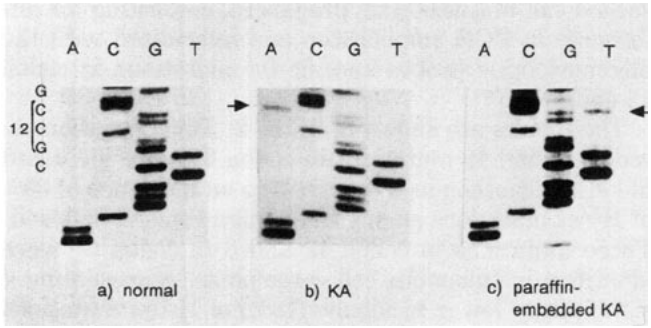

FIGURE 4. Direct sequence of amplified H-*ras* first exon from human keratoacanthomas. Region around codon 12 from normal skin (*a*), freshly obtained tumor (*b*), and paraffin-embedded tissue (*c*) is shown. The antisense primer used for the *in vitro* amplification was the sequencing primer. Arrows indicate bands corresponding to mutated base pairs.

its presence might be a deterrent to progression and therefore selected against during malignant conversion.

The induction of differentiation by *ras* oncogenes has been previously reported by several groups including ours in the PC12 system, where the introduction of a *ras* oncogene or its product triggers neuronal differentiation mimicking the action of nerve growth factor (*42–44*). These observations would indicate that these genes can play a complex role in the biology of the cell, and depending on the system or the particular conditions of the tissue, they could induce proliferation or differentiation.

Another interesting result from these studies is the pattern of H-*ras* mutation in rabbit and humans. In rabbits the only mutation essentially detected is the A:T to T:A transversion in the second position of codon 61 that changes glutamine to leucine. This is the mutation previously reported in murine skin tumors induced by DMBA (*4,36*), and therefore this seems to be an specific target for DMBA that can be conserved through different species. When the mutations in human keratoacanthomas are analyzed, the results indicate that although several other mutations are found, still the most prevalent lesion is that detected in rabbits. This suggests two possible explanations. On one hand, it could be interpreted as an indication that the unknown agent responsible for triggering human keratoacanthomas might have a chemical action similar to DMBA. Alternatively, and more likely, the leucine substitution in position 61 of H-*ras* might have higher potential in skin to induce proliferation, at least in the initial phases of keratoacanthoma development.

From the technical side, it is important to note that mutation studies can be successfully undertaken with minimal archival material. Not only can the DNA contained in one 5-μm slice be amplified by PCR and studied by oligonucleotide mismatch hybridization, but also we show that the material can often be good enough for sequencing, an even more accurate determination of the primary structure of the gene of interest. This allows a comparison between the intensity of the bands for the bases representing the mutated and the normal sequence, providing an indication of the proportion of cells in the tumor with a mutated oncogene.

The combination of PCR amplification and DNA sequencing was also the key to identify the new 146 mutation found in one of the neutron-induced thymic lymphomas, where the correlation between the biological assay (nude mouse tumorigenesis) and the molecular findings (mismatch hybridization and sequence) was clearly apparent.

The common thread linking the studies presented in this paper is the frequent activation of *ras* oncogenes in benign and malignant tumors, not only in experimental systems of carcinogenesis, but in the human tumors for which the animal systems serve as models.

Additionally, the activation of the *ras* oncogene in benign and self-regressing tumors and the fact that only a fraction of malignant tumors contain activated *ras* indicate that other events are necessary to fully complete the progression to malignancy.

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